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13. Abstract *(Maximum 200 Words)* (abstract should contain no proprietary or confidential information)
In order to identify and characterize additional novel or unexpected proto-oncogenes that, in addition to fibroblast growth factors (Fgfs) cooperate with *Wnt1* in murine mammary tumorigenesis, we have generated MMTV-infected *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice. In the first two models, the Wnt oncogenic signal is constitutively overexpressed in their mammary gland, cooperative oncogenic Fgf signals should be abolished by the expression of a dominant-negative FGF receptor (*Fgfr2DN*). The *Wnt1/Fgf3* model displays constitutive overexpression of both *Wnt* and *Fgf* oncogenic signals. In all three models, only those cells carrying MMTV-insertionally activated cellular proto-oncogenes, other than Wnts and Fgfs, should have a growth advantage in the bitransgenic mammary gland. The clonal expansion of these cells leads to mammary tumorigenesis. As proposed, we have generated cohorts of 20-25 MMTV-infected *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic females. As controls, we have also generated uninfected bitransgenic cohort, as well as MMTV-infected and uninfected monotransgenic female control groups. To date, multiple mammary adenocarcinomas have appeared in the MMTV-infected bitransgenic animals. These tumors appeared with a mean latency of 5.4 and 3 months in *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* females respectively. *Wnt10b/Fgfr2DN* tumor histopathology corresponded to papillary lobular, ductal, and metaplastic invasive carcinomas. *Wnt1/Fgfr2DN* tumors were mainly papillary carcinomas, and *Wnt1/Fgf3* tumors displayed features of highly metastatic (to lungs) papillary carcinomas. At least 10 *Wnt10b/Fgfr2DN*, 6 *Wnt1/Fgfr2DN* and 1 *Wnt1/Fgf3* tumors carry newly integrated MMTV proviruses. Our current efforts are to clone and identify these genes, and we are also screening for additional candidate tumors.

14. SUBJECT TERMS
mammary tumorigenesis, mouse mammary tumor virus (MMTV), retroviral insertional mutagenesis, cooperating oncogenes, transgenic mice

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INTRODUCTION

Mouse mammary tumor virus (MMTV) insertional mutagenesis in transgenic mice is a very useful approach to identify novel or unexpected proto-oncogenes implicated the development of mammary tumors. The mouse mammary tumor virus (MMTV) is biological carcinogen that induces murine mammary tumorigenesis by the mechanism of insertional mutagenesis (1-4). MMTV proviral integration in the vicinity of cellular proto-oncogenes may result in activation of their expression. This event confers a selective growth advantage to the mutated mammary epithelial cells and facilitates their clonal outgrowth, eventually leading to tumor formation. In tumor DNAs containing newly integrated proviruses, MMTV is physically linked to adjacent insertionally activated proto-oncogenes, thus it can be used as a molecular tag that facilitates the cloning and identification of the activated gene. Analysis of the MMTV integration loci in mammary tumors from MMTV-infected *Wnt1* or *Fgf3*-transgenics has revealed preferential activation and expression of either *Fgf* genes (*Fgf3, Fgf4, Fgf8*) in *Wnt1* transgenics or activation of *Wnt* genes (*Wnt1, Wnt10b*) in *Fgf3* transgenics (6-11). This fact, together with the decreased tumor latencies observed in *Wnt1/Fgf3* bitransgenics, demonstrate that the activation of *Wnt* and *Fgf* genes and the strong oncogenic cooperation between both growth factor families are crucial events involved the molecular basis of multistep mammary tumorigenesis (12). Nonetheless, little is known to date about additional genes that collaborate in this malignant process.

We propose to use MMTV-insertional mutagenesis in *Wnt10b/Fgfr2DN* bitransgenic mouse models, to identify and characterize novel or unexpected proto-oncogenes that, in addition to *Fgfs*, cooperate with *Wnts* in multistep mammary tumorigenesis. In these mice, the Wnt oncogenic signal will be constitutively overexpressed in the mammary gland (13,14). However, cooperative oncogenic Fgf signals will be abolished by the expression of a truncated form of the fibroblast growth factor receptor 2 (*Fgfr2DN).* The modified receptor functions in a dominant-negative fashion, thus blocking Fgf signaling mediated by endogenous Fgfrs (15-19). We expect that only those cells carrying insertionally activated cellular proto-oncogenes other than Wnts and Fgfs will have a growth advantage in the bitransgenic mammary gland. Eventually, the clonal outgrowth of these cells should give rise to mammary tumors with an accelerated latency compared to that in infected non-transgenic littermates. These can be then analyzed, using an MMTV-molecular tagging approach, to isolate and identify the activated proto-oncogenes.

In order to ensure that we have access to enough number of potential candidate tumors in which we can conduct our insertional mutagenesis and oncogenic cooperation studies, I also proposed to create two additional bitransgenic mouse models: *Wnt1/Fgfr2DN* and *Wnt1/Fgf3*. Moreover, it is also possible that the variation in the constitutive *Wnt* and *Fgf* overexpression pattern of each experimental group may lead to the identification of a wider range of novel or unexpected cooperating oncogenes. The working hypothesis and rationale originally postulated, applies to the *Wnt1/DNFgfr2* experimental group as well. In a similar fashion, in MMTV-infected *Wnt1/Fgf3* bitransgenics, we expect that constitutive overexpression of *Wnt1* and *Fgf3* oncogenic signals will lead to the genesis of clonal mammary tumors displaying the insertional activation of oncogenes that cooperate with both *Wnts* and *Fgfs* in multistep mammary tumorigenesis.

The original specific aims of this project are:

I. Generation of MMTV-infected *Wnt10b/Fgfr2DN* bitransgenic mice.
II. Isolation and identification of novel or unexpected proto-oncogenes insertionally activated in tumors of MMTV-infected *Wnt10b/Fgfr2DN* bitransgenic mice.
III. Analysis of the oncogenic potential of the identified proto-oncogene, and demonstration of its cooperativity with *Wnt* genes in cell transformation *in vitro*.

REPORT BODY

The basic experimental design that was followed in this project is described in figure 1. Briefly, single transgenic mice were mated to generate enough numbers of positive bitransgenic or single transgenic females. For each bitransgenic mouse model, we created 3 cohorts (n=40-50 mice/cohort) of female mice: a bitransgenic cohort, and one cohort for each single transgene type, as controls. Half of the females (n=20-25) in each cohort were infected with MMTV at 3-4 weeks of age. The other half remained uninfected as negative controls. In order to stimulate ongoing mammary epithelial cell division, and hence MMTV infection spreading and expression, all females were uninterruptedly bred and allowed to lactate during 7-10 days after each pregnancy until mammary tumors were detected. The tumor DNAs were then analyzed for the presence of new MMTV integrations. Tumors DNAs carrying such integrations were used to generate viral-cellular junction fragments which will be used to clone insertionally activated genes by inverse polymerase chain reaction (IPCR).

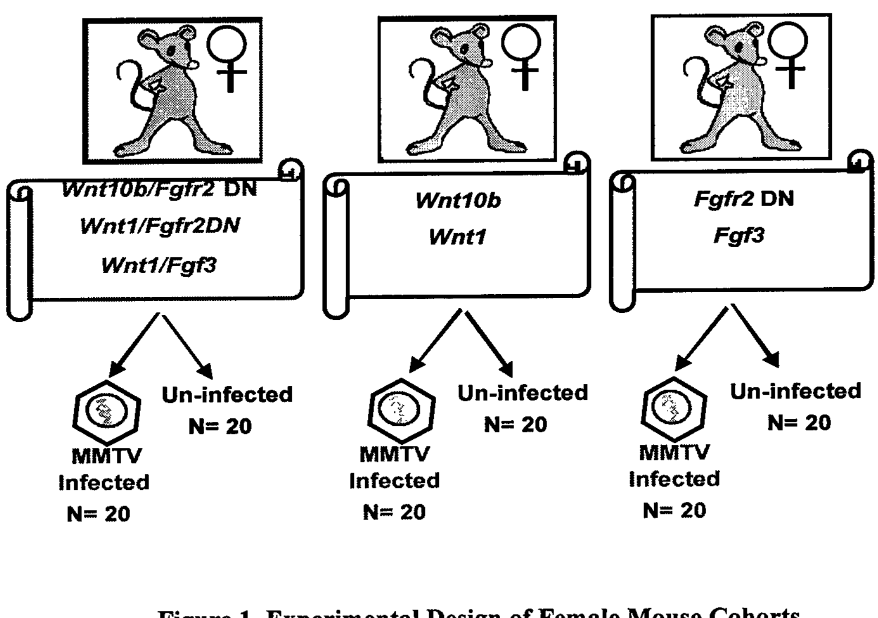

Figure 1. Experimental Design of Female Mouse Cohorts

Task 1. Generate MMTV-infected _Wnt10b/Fgfr2DN_ bitransgenic mice (months 1-5)

Task 1B. Generate MMTV-infected _Wnt1/Fgfr2DN_ and _Wnt1/Fgf3_ bitransgenic mice (months 12-24)

A. Establish matings between _Wnt10b_ and _Fgfr2DN_ single transgenics
B. Southern blot analysis of positive transgenic offspring

We created *Fgfr2DN* transgenic mice which strongly express the transgene in the mammary gland (not shown). These *Fgfr2DN* transgenic mice were bred to *Wnt10b* transgenic mice (C57BL6xSJL/J) that had been created in our laboratory or to a purchased *Wnt1* male mouse (The Jackson Lab, B6SJL[*Wnt1*]HeV). The purchased Wnt1 transgenic was also mated to two *Fgf3* transgenic females mice (inbred FVB/N; TG.NR line) kindly provided by Dr. Philip Leder. The *Fgfr2DN* transgene is designed to express a truncated Fgfr2 protein (ie, the extracellular and transmembrane regions only without the cytoplasmic tyrosine kinase domain) which acts in a dominant-negative fashion to inhibit the activity of normal FGF receptor proteins in the cell. The *Wnt10b* mice generated in our lab express a transgene that cointains the mouse *Wnt10b* gene driven by the MMTV LTR enhancer. The *Wnt1* mice express a transgene containing the mouse *Wnt1* gene driven by the MMTV LTR enhancer. The *Fgf3* mice carry a transgene with the murine *wild-type Fgf3* cDNA under the control of a truncated MMTV LTR (lacking sequence 5' to the *Cla*I site) and with SV40 transcriptional processing signals.

Offspring from these matings were screened by Southern blot analysis of genomic tail DNA (Fig. 2, 3). The presence of the *Wnt10b* transgene was detemined following restriction enzyme digest with *BamHI* restriction enzyme digest and blot probing with a *Wnt10b* cDNA fragment. Similarly, *Wnt1* transgenics were screened with *BamHI* and a *Wnt1* cDNA probe. *Fgf3* and *Fgfr2DN* transgenics were detected upon *HindIII* digest and hybridizing with the appropriate probe. The expected sizes of transgene-specific bands were: *Wnt10b*, 2.5 Kb+4.5 Kb bands; *Wnt1*, 4.0 Kb+2.3 Kb bands; *Fgf3*, 1.8 Kb band; *Fgfr2DN*, 2Kb band.

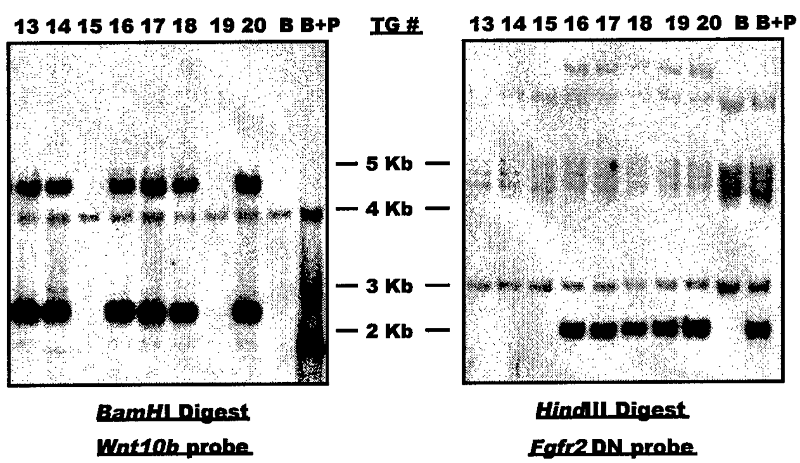

Figure 2. Southern Blot Screening of _Wnt10b/Fgfr2DN_ bitransgenic mice. Left panel: *Wnt10b* transgenic mice display 2.5 and 4.5 Kb bands. **Right panel**: *Fgfr2DN* transgenic mice display a 2.0 Kb band. Numbers on top indicate transgenic mouse identification number. Molecular weight markers are displayed between the panels.

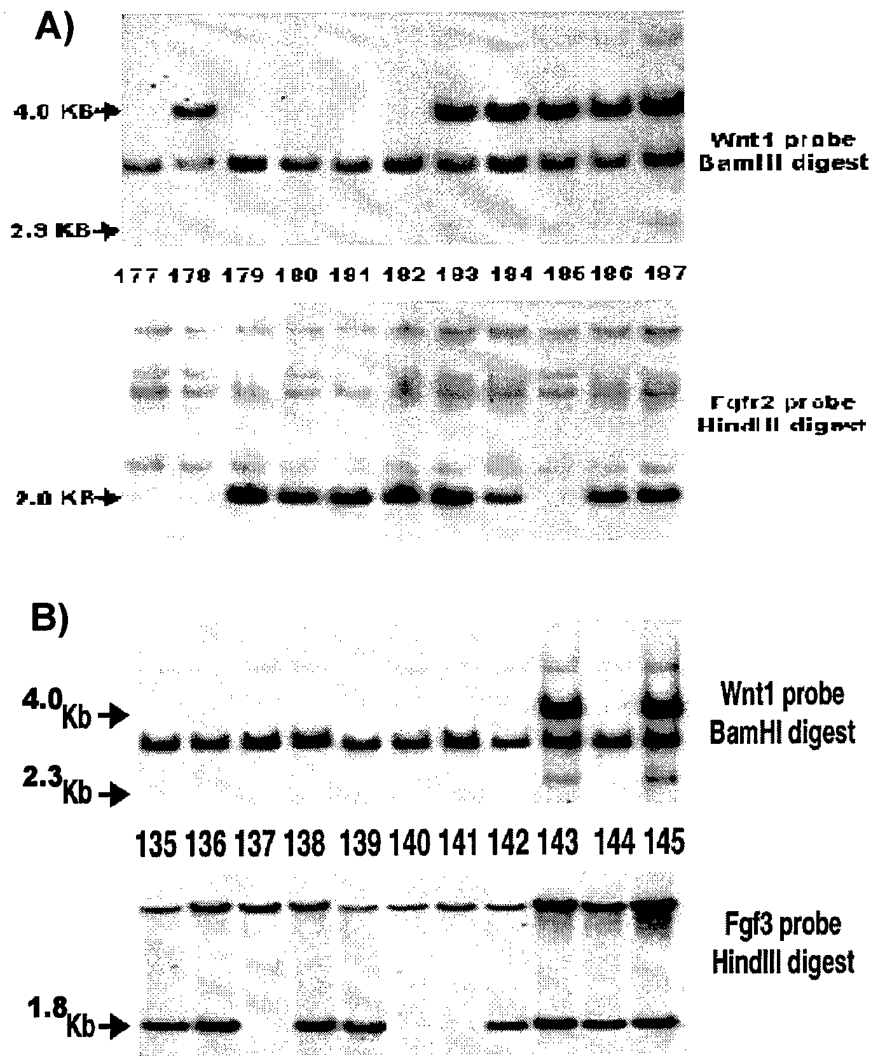

Figure 3. Representative Southern blot screening of *Wntl/Fgfr2DN* and *Wntl/Fgf3* mice. Tail DNAs were digested with *BamH*I or *Hind*III and probed with *Wntl, Fgfr2* or *Fgf3* cDNA respectively. *Wntl* transgenics display 2.3 Kb and 4.0 Kb bands. *Fgfr2DN* transgenics display a 2.0 Kb band. *Fgf3* transgenics display a 1.8 Kb band. *Wntl/Fgfr2DN* transgenics (#'s 183, 184, 186, 187) and *Wntl/Fgf3* bitransgenics (#'s 143, 145) display all characteristic bands.

For each mouse model study, 3 female cohorts (n=40-50 each) were generated: 1) *Wnt/Fgf* or *Wnt/Fgfr2DN*, 2) *Wnt*, and 3) *Fgf* or *Fgfr2DN*, (Fig. 1). A total of 313 offspring from *Wnt10b/Fgfr2DN* matings, 308 offspring from *Wntl/Fgfr2DN*, and 260 offspring from *Wntl/Fgf3* matings were screened in this manner.

C. Grow MMTV*supF* XC rat cells.
D. Infect, via intra-peritoneal (IP) injection, bitransgenic females and non-transgenic littermates.

<u>*Task 2.*</u> **Obtain mammary tumor tissue and nucleic acids from MMTV-infected *Wnt10b/Fgfr2DN* bitransgenic mice (months 6-11)**

<u>*Task 2B .*</u> **Obtain mammary tumor tissue and nucleic acids from MMTV-infected *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice (months 12-24)**

A. **Conduct weekly physical examination of the mice to detect the development of mammary tumors**
B. **Surgically resect, under anesthesia, the mammary tumors**
C. **Process tumor samples for histopathology and tumor DNA and RNA analysis**

<u>*Task 3.*</u> **Identify MMTV-proviral-cellular DNA junction fragments and clone the cellular sequences contained in them (months 12-24)**

We infected with MMTV half of the females from each experimental cohort at 3-4 weeks of age, while the other half remained uninfected. XC rat sarcoma cells, producing a hybrid MMTV provirus, composed of the 5'-half from *Mtv1* and a 3'-half from C3H MMTV, and carrying a bacterial *supF* gene (Jiang 1999), were initially used for the infection of the *Wnt10/Fgfr2DN* females and corresponding single transgenic controls. Stably transfected rat XC cell clones, that produce different hybrid MMTV variants, were previously generated in my mentor's lab. Prior to injection, the levels of MMTV expression in these clones were characterized by northern blot analysis of their total RNA (20 μg) after growth in the presence of dexamethasone, a glucocorticoid that stimulates MMTV expression. The expression level of MMTV in the EH-*supF*9 clone was considered suitable for our experiment, when compared to the expression level of *wild-type* MMTV(C3H)-expressing XC clones. The EH-*supF*9 clone was further expanded and used for MMTV infection (*via* intraperitoneal injection) of our mice.

During a seven month-long period following infection with MMTV-EH *supF*9, only two *Wnt10/Fgfr2DN* females developed mammary tumors, and only of these (# 136) contained new MMTV integrations. This represents a much lower incidence than we initially expected. Unrelated experiments done by others in my mentor's lab suggest that our *Wnt10b* mice may be losing transgene expression overtime. This fact may be contributing to the low tumor incidence, and somehow prolonged latency, that we have observed in our bitransgenic animals. In addition, we have also considered the possibility that our mice may not be efficiently infected with the retrovirus, due perhaps to some unknown immune-compatibility problems between host and virus that could be associated with the dependence of MMTV infection on B and T-cell activation and expansion. In order to circumvent this unforeseen potential problem, our bitransgenic mouse cohort was re-injected with the *wild-type* MMTV(C3H)-producing Mm5MT mouse cell line. This *wild-type* virus has been reported to strongly infect and induce mammary tumor formation in mouse strains similar to ours.

Approximately between one to two months after the new infection, 50% of the females developed very large mammary tumors always around the injection area. Some of these animals also developed massive intra-abdominal tumor masses, and 2/3 of all the injected animals

eventually succumbed to them. The very short latency and the locations of the new tumors suggested that the carrier Mm5MT cell may have not been rejected by the infected animals and that the tumors may have developed not from host cells insertionally mutated by MMTV(C3H), but as the result of unwanted C3H/Mm5MT spreading and proliferation instead. Rejection of Mm5MT cells was expected because the H2 (mouse major histocompatibility complex) haplotype of the Mm5MT cells (*k* haplotype) was different from that of the host mouse strains (*d, b* and/or *s* haplotypes). In order to test the possibility that the tumors arose from Mm5MT cells, the tumor DNAs were digested with *Eco*RI or *Hind*III and analyzed by Southern blot and hybridization to an MMTV-Env probe. Tumors of clonal origin arising from MMTV-infected mammary epithelial cells usually contain very few newly integrated proviruses. On the other hand, the injected Mm5MT cells—and any tumors arising from them—contain hundreds of MMTV proviruses, integrated at different loci. Therefore, while tumor DNAs from the former tumors should display only a few MMTV-specific bands on a Southern blot, DNAs from the latter tumors will display a smear of multiple-size bands. Our analysis did indeed confirm this second possibility (Fig. 4), since several tumors (mammary and intra-abdominal) displayed a smeared band pattern. We therefore considered that all the tumors which quickly arose after the second round of infection were not good candidates for our retroviral insertional mutagenesis studies.

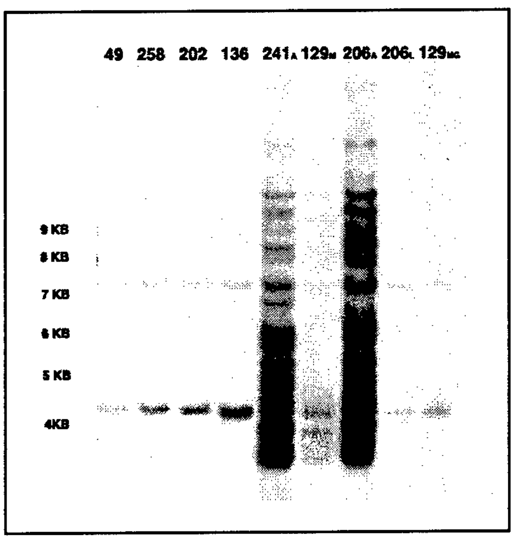

Figure 4. Southern blot analysis of tumor origin in *Wnt10b/Fgfr2DN* females infected with XC cells (MMTV EH-supF9) versus Mm5MT cells (MMTV C3H). DNAs from tumors arising from Mm5MT cells display a band smear corresponding to hundres of proviruses present in these cells. A: abdominal tumor. M: mammary tumor. L: liver. MG: mammary gland. *EcoRI* digest, MMTV-Env probe. Molecular weight markers are displayed on the left

Out of the remaining surviving *Wnt10b/Fgfr2DN* females (n=9), five of them eventually developed a total of ten independent mammary tumors with variable postinfection latencies. Southern blot analysis demonstrated the presence of new proviral integrations in eight of these tumors (Fig. 5). At the end of out *Wnt10b/Fgfr2DN*, we were thus able to collect a total of 10 tumors valid for further study. Half of these tumors (#'s 64_{L1}, 64_{L5}, 74_{R1}, 136, 72) contained new hybrid MMTV proviruses, while the other half (#'s 74_{L5}, 207_{L1+L2}, 207_{R1}, 207_{R2}, 207_{R3}) contained both hybrid and C3H proviruses. Macroscopically, the tumors were solid, well encapsulated masses. The histopathology of these tumors was predominantly of the papillary and lobular mammary adenocarcinoma type (Fig. 6). No distant metastases were observed in the affected animals.

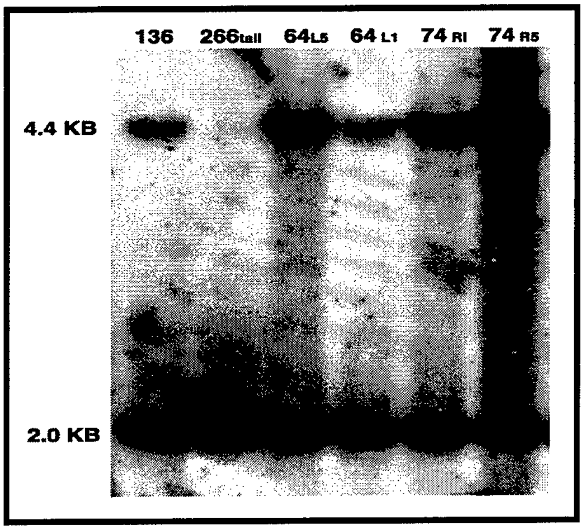

Figure 5. New proviral integrations in mammary tumors from MMTV-infected *Wnt10b/Fgfr2DN*. Tumor DNAs (number indicated on top of each lane) were digested with *Bgl*II and blots were probed with an MMTV *BglII-BglII* 4.2 Kb probe. A 2.0 Kb band corresponds to endogenous proviruses, while a 4.4 Kb band indicated the presence of newly integrated proviruses. 266_{tail}: negative control (tail DNA from uninfected *Wnt10b/Fgfr2DN* female).

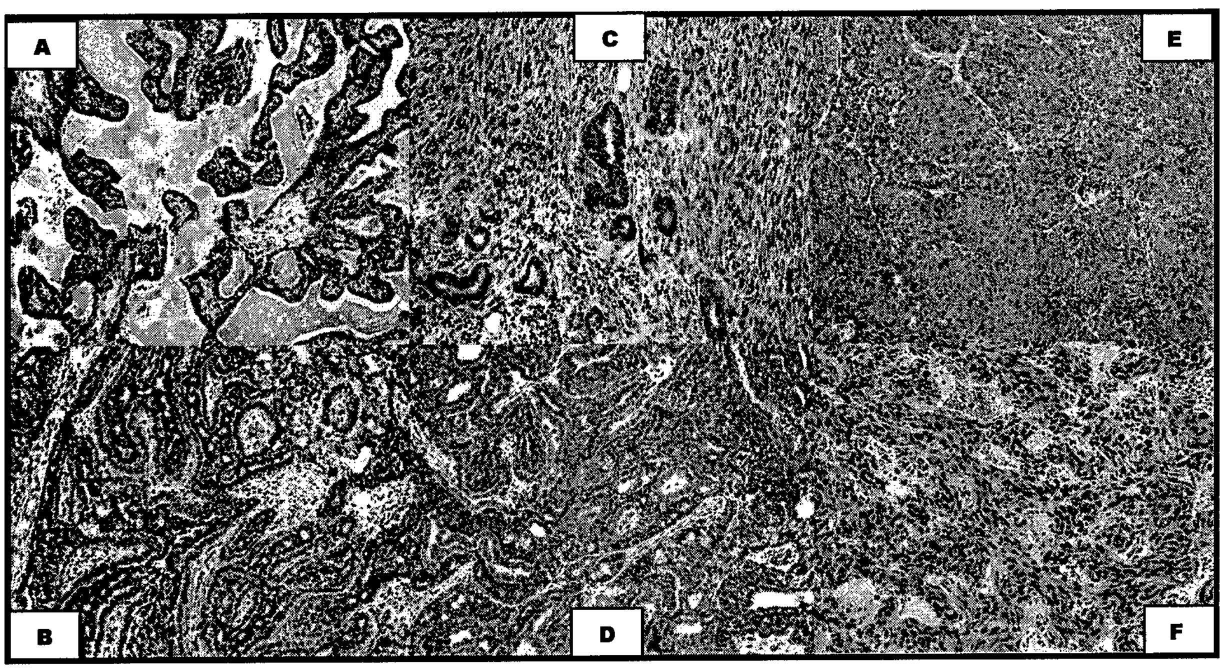

Figure 6. Mammary tumor histopathology in MMTV-infected *Wnt10b/Fgfr2DN* and *Wnt1/Fgfr2DN* females. Hematoxylin-Eosin stain. A) Papillary mammary carcinoma, probably invasive. B) Solid type papillary mammary carcinoma, invasive. C) Ductal mammary carcinoma, invasive. D) Tubular mammary carcinoma. E) Lobular carcinoma, alveolar type, invasive. F) Metaplastic carcinoma, spindle type. A, C, E, F: *Wnt10b/Fgfr2DN* tumors. B, D: *Wnt1/Fgfr2DN* tumors.

In order to identify rearranged viral/cellular junction DNA fragments that could be used for cloning and further study, we analyzed the 10 tumors DNAs by Southern blot, after *Xho*I restriction ezyme digest, using an MMTV-Gag probe. As shown in Figure 7, multiple rearranged fragments could be detected in all *Wnt10/Fgfr2DN* mammary tumor DNAs.

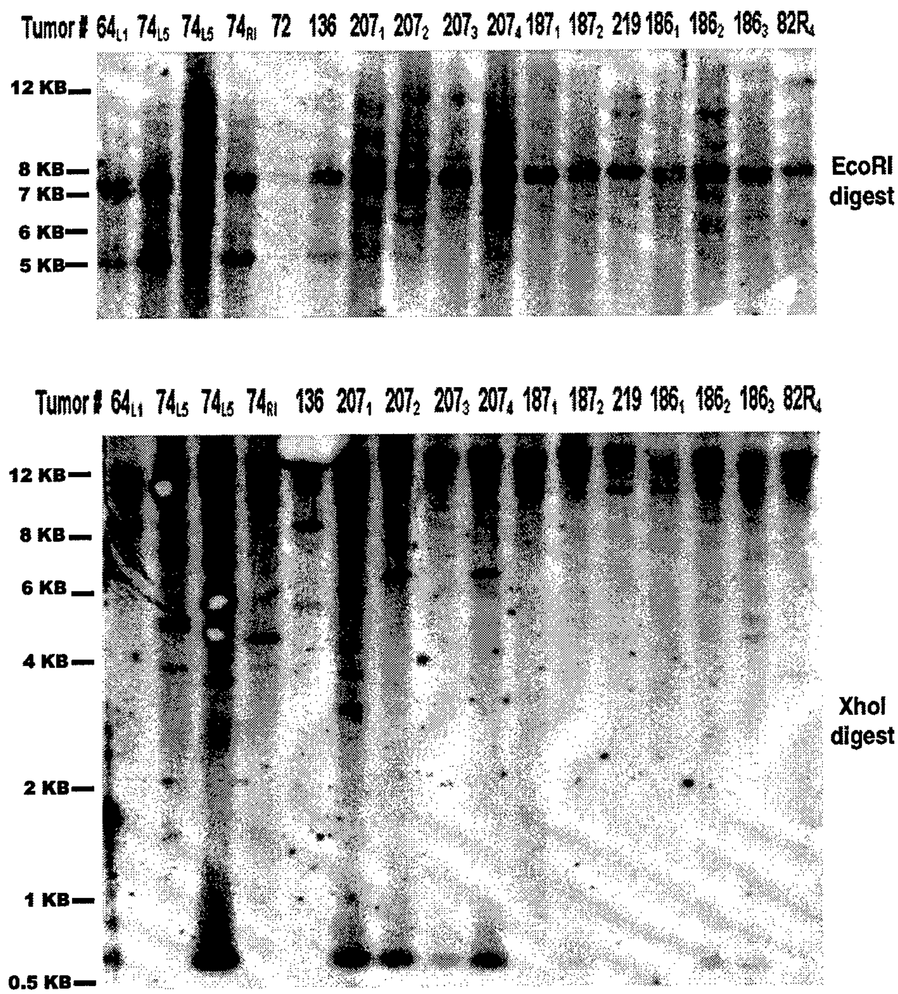

Figure 7. New MMTV proviral insertions in mammary tumors from MMTV-infected transgenic mice. The panel shows the Southern blot analysis of genomic DNAs isolated from mammary tumors of MMTV-infected *Wnt10b/Fgfr2DN* (# 64_{L1} to 207_4), *Wnt1/Fgfr2DN* (# 187_1 to 186_3), and *Wnt1/Fgf3* transgenic mice (# 82 R_4). All DNAs display large MMTV fragments, indicative of endogenous retroviruses in these mice. Most of the tumor samples have additional rearranged fragment(s), indicating the presence of clonal, tumor-specific newly integrated MMTV proviruses. DNAs were digested with *EcoRI* (top) or *XhoI* (bottom). ^{32}P-labeled MMTV *gag* cDNA was used as a probe.

MMTV-insertional mutagenesis in *Wntl/Fgfr2DN* and *Wntl/Fgf3* bitransgenic mice. Half of the females in the *Wntl/Fgfr2DN* and *Wntl/Fgf3* mouse cohorts, as well as the corresponding single transgenic control females, were injected with purified, cell-free *wild-type* MMTV(C3H). This viral strain is highly pathogenic in our mouse strain and was chosen with the hope to avoid any immune incompatibility-related problems, as well as to increase the number of mammary tumors produced in our animals.

Currently, 16 out 19 MMTV-infected *Wntl/Fgfr2DN* females have developed mammary tumors, with a median latency of 5.4 months and tumor load values between 1–4 tumors per animal (Fig. 8). Tumor latencies for the control groups were as follows: uninfected *Wntl/Fgfr2DN*, 3.6 months; infected *Wnt1*, 4.8 months; uninfected *Wnt1*, 3.9 months; infected *Fgfr2DN*, 9.8 months; uninfected *Fgfr2DN*, no tumors developed. Southern blot analysis of mammary tumor DNAs revealed the presence on new proviruses in 4 of the tumors (Fig. 9). Rearranged cellular-viral junction fragments could be detected as well in these tumors by Southern blotting (Fig. 7).

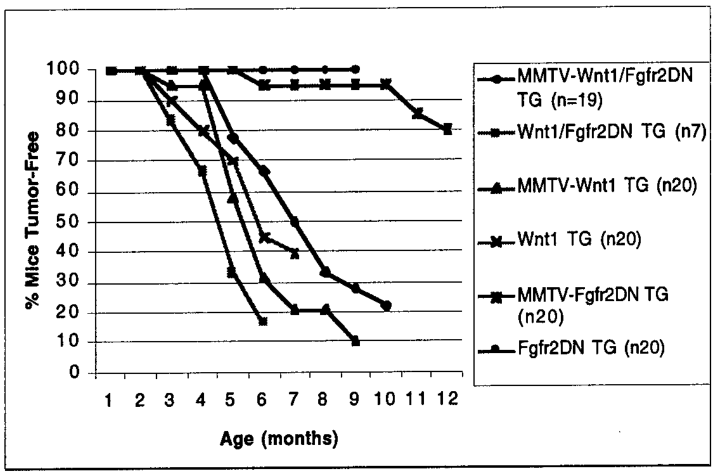

Figure 8. Incidence of mammary tumors in *Wntl/Fgfr2DN* female mice and control cohorts. The percentage of animals in each cohort remaining free of palpable tumors was plotted at monthly intervals as a function of age. The number of animals in each group are indicated in parentheses.

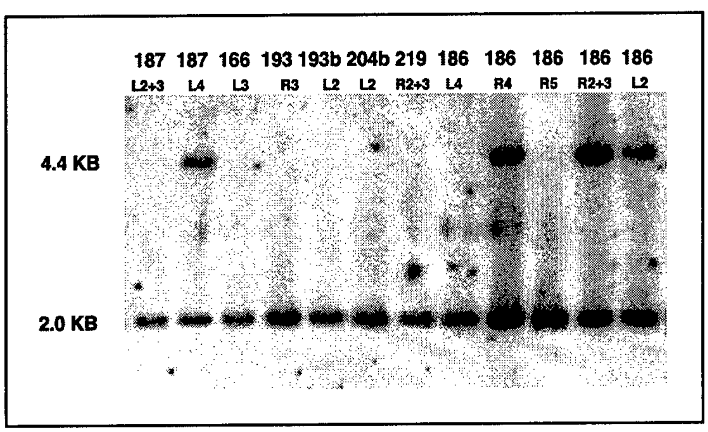

Figure 9. New proviral integrations in mammary tumors from MMTV-infected *Wntl/Fgfr2DN* females. Tumor DNAs (number indicated on top of each lane) were digested with *Bgl*II and blots were probed with an MMTV *BglII-BglII* 4.2 Kb probe. A 2.0 Kb band corresponds to endogenous proviruses, while a 4.4 Kb band indicated the presence of newly integrated proviruses.

Upon necropsy examination, mammary tumors varied from a solid, well encapsulated mass to well defined cystic and very necrotic multilobulated tumors. Lung metastases were macroscopically manifest in 5 animals, and hepato-splenomegaly was observed in 8 animals (Fig. 10). Histologically, mammary tumors were invasive papillary carcinomas (Fig. 6).

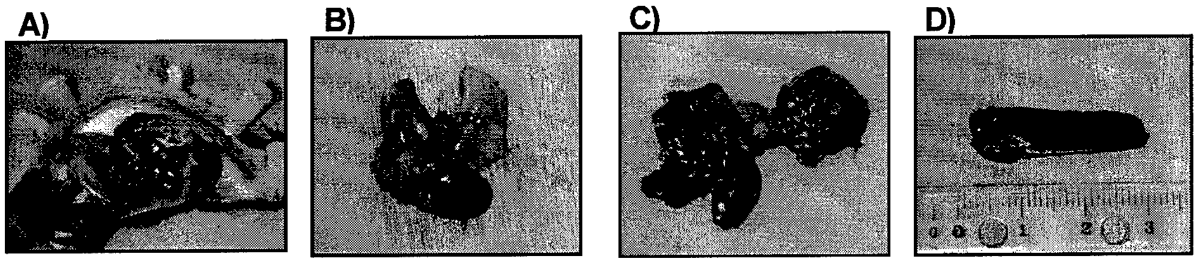

Figure 10. Macroscopic pathology necropsy findings in MMTV-infected *Wnt1/Fgfr2DN* transgenic females. A) Mammary tumor (L_1+L_2 location). **B)** Normal lungs. **C)** Massive bilateral lung metastases. **D)** Splenomegaly.

In comparison to the mammary tumors observed in MMTV-infected *Wnt1/Fgfr2DN* females, necropsy examination of *Wnt1/Fgf3* females showed all glands to be affected to different degrees, varying from focal or diffuse hyperplastic nodules to generalized mammary tumors. Common necropsy findings were lung metastases and hepato-splenomegaly (Fig. 11, 12).

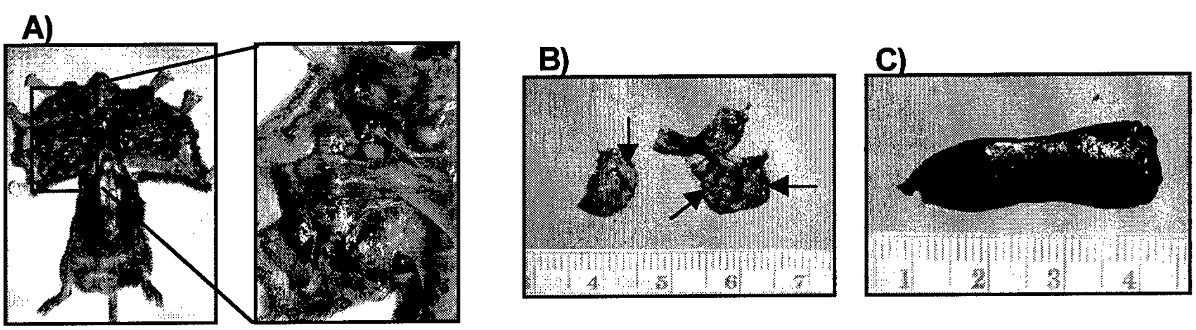

Figure 11. Representative necropsy macroscopic findings in MMTV-infected *Wnt1/Fgf3* females. A) Generalized mammary tumors & magnification (right). B) Lung metastases (solid arrowhead). C) Splenomegaly (cm scale)

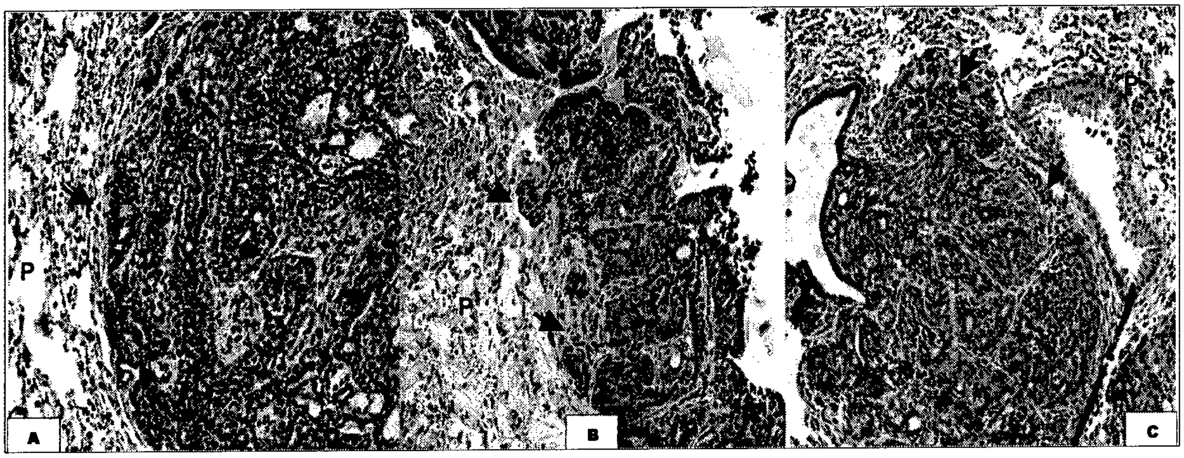

Figure 12. Lung metastases (papillary mammary carcinoma) from *Wnt1/Fgf3* mouse mammary tumors. Hematoxylin-Eosin stain. A, B, and C each correspond to a different bitransgenic animal. T: Mammary tumor metastasis. P: Pulmonary parenchyme. Solid arrow: Tumor/lung parenchyme boundary.

In the MMTV-infected *Wnt1/Fgf3* group, mammary hyperplastic glands could be observed in all animals approximately one month after MMTV infection. The hyperplasia was pregnancy-dependent in the majority of cases, and was also observed in uninfected *Wnt1/Fgf3* female controls. Seventeen out of nineteen MMTV-infected bitransgenic females have developed mammary tumors to date. The median tumor latency is 3 months, and tumor load values range from 1 to 6 independent tumors per animal (Fig. 13). Data for uninfected bitransgenic controls is not available at present. Median tumor latency values for other control groups were as follows: infected *Wnt1*, 4.8 months; uninfected *Wnt1*, 3.9 months; infected *Fgf3*, 6.7 months; uninfected *Fgf3*, 9.2 months.

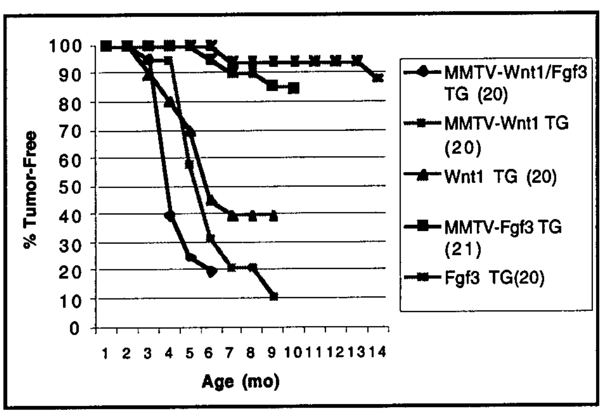

Figure 13. Incidence of mammary tumors in *Wnt1/Fgf3* female mice and control cohorts. The percentage of animals in each cohort remaining free of palpable tumors was plotted at monthly intervals as a function of age. The number of animals in each group are indicated in parentheses.

Southern blot analysis of *Wnt1/Fgf3* mammary tumor DNAs from MMTV infected females was negative for the presence of newly integrated proviruses as well as for rearranged viral-cellular junction fragments. This finding suggests that the tumors developed so far in these animals are the result of the cooperative oncogenic effect of the *Wnt1* and *Fgf3* rather than a consequence of mammary tumor acceleration due to MMTV-induced insertional activation of additional oncogenic collaborators.

DISCUSSION and SUMMARY

The discovery, more than twenty years ago, of *Wnt1*--the first proto-oncogene directly implicated in mouse mammary tumorigenenesis--as a preferential target for MMTV proviral tagging insertions, opened the door to studies in the field of retroviral insertional mutagenesis and oncogenic cooperation in murine breast cancer. Since then, it has become clear that the collaborative/synergistic effects of specific genetic mutations play a key role in mammary tumorigenesis and progression. Among these genetic events, it is now well established that mutations leading to the activation and cooperation of Fgf and Wnt signaling are key early events involved in the process.

In order to identify oncogenes other than *Fgfs* that cooperate with *Wnt* genes in multistep mammary tumorigenesis, we have created three new bitransgenic mouse models of breast cancer: *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN*, and *Wnt1/Fgf3* transgenic mice. In all three, either through concomittant overexpression of transgenic Wnt and Fgf signals (*Wnt1/Fgf3* model), or through activation of the former and blocking of the latter (*Wnt10b/Fgfr2DN* and *Wnt1/Fgfr2DN* models), MMTV-mediated insertional mutagenesis of genes other than *Wnts* and *Fgfs* should give rise to the formation of clonal tumors with an accelerated latency.

***Wnt10b/Fgfr2DN* bitransgenics.** Our *Wnt10b/Fgfr2DN* mice were initially infected with a hybrid MMTV provirus, which, despite its proven mammary tumorigenicity, did not lead to the appearance of hybrid MMTV-infected mammary tumors but in 1/25 of the females infected. Efficient MMTV infection and spreading to the mammary gland depends on the immune compatibility between the MMTV strain and the host strain (specific lymphoid MHC II receptor type). Certain inbred mouse strains (e.g., C57BL6) are resistant to early, but not late, MMTV infection due to MHC II incompatibility, whereas others (e.g., BALB/c) are hightly sensitive to infection and tumorigenesis. The *Wnt10b/Fgfr2DN* mice have a mixed C57BL6, SJL/J, and BALB/cByJ background. It is thus possible that the expression of an unknown genetic modifier in this mixed strain may lead to resistance to infection or tumorigenesis by the hybrid MMTV strain. In order to circumvent this potential caveat, the bitransgenic females were reinfected with mouse *wild-type* MMTV(C3H)-producing Mm5MT cells. This viral strain is highly pathogenic in a wide variety of mouse strains. To our surprise, the mice rapidly (<2 mo) developed very aggressive and disseminated tumors, which we determined were of Mm5MT origin. Out of the surviving animals in this infected cohort, 10 independent mammary tumors arose several months later. Due to the dual time of infection and the reduction of group size, an estimation of tumor latency was not determined in these mice. The pathology report on the mammary tumors produced in these animals revealed a variety of tumor types: papillary lobular, ductal, and metaplastic invasive carcinomas. The implications of the tumor type heterogeneity in terms of its relation to activation of specific proto-oncogene types remain unclear at this moment and should require further study. All 10 of the tumors from surviving animals contained new MMTV insertions (five containing hybrid provirus, and five with new MMTV(C3H)+hybrid proviral integrations). Southern blot analysis of the tumor DNAs revealed the existence of multiple rearranged viral-cellular junction fragments in all of the tumors. These fragments are candidates to harbor potential insertionally activated proto-oncogenes, and are currently being isolated using various long inverse PCR approaches.

***Wnt1/Fgfr2DN* bitransgenics.** In order to optimize MMTV-infection efficiency while avoiding the problems that we encountered in the *Wnt10b/Fgfr2DN* project, our *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* mice were infected with purified wild-type MMTV(C3H) virus directly. All MMTV-infected *Wnt1/Fgfr2DN* females have developed mammary tumors, most of them corresponding to papillary carcinomas. Despite the high tumor incidence, only 4 tumors contained newly integrated proviruses. The observed tumor latency in these animals was 5.4 months. Surprisingly, this constitutes a higher value than that observed in the uninfected *Wnt1/Fgfr2DN* control groups (3.6 months). The difference in sample size between both groups (19 vs. 7, respectively) is significant at the moment, and the latency value for the controls may not be truly representative. Another possibility that may explain the unexpected delay in tumor formation in the infected bitransgenics is that the MMTV-infection status may modify the action of certain effectors involved in the control of mammary proliferation. To this respect, for example, it has been reported that MMTV-infected mice are more susceptible to stimulation by progesterone, whereas those without the virus respond more to beta-estradiol. The proliferative response of the mouse mammary gland to ovarian hormones, and perhaps other factors, can thus be modified by mammary tumor virus infection and may affect the incidence of mammary tumor formation in our model. Nevertheless, the tumors generated thus far with MMTV insertions, together with future such tumors, will be used to search for insertionally activated proto-oncogenes.

***Wnt1/Fgf3* bitransgenics.** MMTV-infected *Wnt1/Fgf3* females developed generalized metastatic mammary papillary carcinomas with high incidence and with an accelerated median latency of 3 months compared to the control groups (infected *Wnt1*, 4.8 months; uninfected *Wnt1*, 3.9 months; infected *Fgf3*, 6.7 months; uninfected *Fgf3*, 9.2 months). Data for uninfected bitransgenic controls is not available at present. It is hence not possible to determine if MMTV-infection results in significant tumor latency shortening which may reflect the occurrence of additional oncogenic activations in these tumors. We have not been able to detect new proviral integrations in any of the tumors developed in these infected bitransgenics so far. We thus suggest that strong expression and oncogenic cooperation between the *Wnt1* and *Fgf3* transgenes is leading to the formation of mammary tumors of generalized tumors well before the effects of MMTV-induced oncogenic activations may be apparent. It is thus possible that this particular model may not be useful for our study purpose, but this conclusion must wait until the tumor kinetics data from the uninfected bitransgenic females are available and from the analysis of additional tumors, which continue to arise. Tumors are now arising that show new gross pathologies (i.e., they are solid, noncystic and apparently clonal) that distinguish them from the surrounding hyperplastic and cystic mammary pathologies common to these mice, so these new tumors will be examined for MMTV insertions and, if appropriate, for activated proto-oncogenes.

KEY RESEARCH ACCOMPLISHMENTS

1. Generation of *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice.
2. MMTV-infection of *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* females, as well as corresponding monotransgenic control groups.
3. Induction of mammary adenocarcinomas in MMTV-infected *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* females.
4. Confirmation of the existence of new MMTV proviral integrations and the clonal origin of the *Wnt10b/DNFgfr2*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* mammary adenocarcinomas.
5. Partial characterization of expression levels for various *Wnt* and *Fgf* oncogenes in the *Wnt10b/DNFgfr2* mammary tumors.
6. Histological analysis of tumor samples.
7. Analyses of *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* tumor load and kinetics.

REPORTABLE OUTCOMES

1. Generation of MMTV-infected *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice
2. Generation of mammary tumors from MMTV-infected *Wnt10b/Fgfr2DN*, *Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice
3. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. December 28, 2000. Avances en Biologia Molecular por Jovenes Investigadores en el Extranjero. Centro Nacional de Biotechnologia, Madrid, Spain.
4. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. November 18, 2000. University of Southern California, Los Angeles, California.
5. Presentation: Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis. February 16, 2001. Childrens Hospital Los Angeles, Los Angeles, California.

6. Mouse models of oncogenic cooperation in breast cancer. December 12, 2001. University of Southern California, Los Angeles, California.

7. Identification of Oncogenes Cooperating in Murine Mammary Tumorigenesis and Transgenic Mouse Models of Breast Cancer. Doctoral Thesis Defense. April 18, 2002. Department of Molecular Microbiology and Immunology, University of Southern California, Los Angeles, California.

CONCLUSIONS

During the past twenty-four months, we have accomplished the generation of MMTV-infected *Wnt10b/DNFgfr2, Wnt1/Fgfr2DN* and *Wnt1/Fgf3* bitransgenic mice. The oncogenic cooperation between members of the *Wnt* and *Fgf* gene families is a crucial and well known molecular event implicated in the development of mammary tumors in mice. The use of MMTV-insertional mutagenesis in a Wnt/DNFgfr model is therefore a logical step when trying to elucidate what additional genetic events are involved. Our MMTV-infected bitransgenic mouse models constitute the first reported contribution to taking to take the multistep mammary tumorigenesis studies one step beyond. To date, multiple mammary tumors have appeared in the MMTV-infected bitransgenics. Molecular analysis of these tumors shows that at least sixteen of them may be potential canditates to harbor insertional activations of novel or unexpected oncogenes other than Wnts or Fgfs. The identification and cloning of such genes is our next immediate goal.

We and others have improved several experimental strategies (see Materials and Methods of original proposal) that will facilitate the cloning of such genes. In addition, we are currently using several inverse PCR-based approaches to clone genomic loci tagged by MMTV insertions and to identify insertionally activated genes that these loci may contain. We are also taking advantage of the recent and upcoming advances made in the completion of the mouse and human genome projects to expedite the identification of the candidate genes. We therefore feel that our overall goal will be successfully achieved in the coming year.

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APPENDICES

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